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Research Article

Effect of Antimicrobial Edible Coatings and Modified Atmosphere Packaging on the Microbiological Quality of Cold Stored Hake *(Merluccius merluccius)* Fillets

X. Carrión-Granda,[1] I. Fernández-Pan,[2] J. Rovira,[3] and J. I. Maté[4]

[1] *Viscofan, S.A. R+D Department, Polígono Industrial Berroa, C/Berroa 15-4°, Tajonar, 31192 Navarra, Spain*
[2] *BCC Innovation, Basque Culinary Center, Paseo Juan Avelino Barriola 101, 20009 San Sebastián, Spain*
[3] *Biotechnology and Food Science Department, Universidad de Burgos, Plaza Misael Bañuelos s/n, 09001 Burgos, Spain*
[4] *Food Technology Department, Universidad Pública de Navarra, Campus Arrosadia s/n, 31006 Pamplona, Spain*

Correspondence should be addressed to I. Fernández-Pan; ifernandez@bculinary.com

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The effect of whey protein isolate (WPI) coatings incorporated with essential oils (EOs) and combined with modified atmosphere packaging (MAP) on the microbiological quality of fresh hake fillets was evaluated. Fresh hake fillets were coated with different formulations of WPI-EO coatings and packaged under air and MAP conditions (50% CO_2/45% N_2/5% O_2). When WPI-enriched films were applied with or without the presence of MAP over hake fillets with a high initial microbial population, a limited but significant effect on the microbial growth was observed. This effect was more intense on *Enterobacteriaceae* and H_2S-producing bacteria. When hake fillets with lower initial microbial counts were treated, a more intense antimicrobial effect was observed and a synergistic effect between WPI-EO coatings and MAP was detected. A significant extension of the lag phase and reduction, primarily, on the total viable counts and H_2S-producing bacteria, was detected, doubling the shelf-life of hake fillets compared with control samples. The initial microbial load of the samples is a key factor influencing the effectiveness of the treatment. The obtained results demonstrated the effectiveness of this combined strategy as a promising alternative for enhancing the microbial quality of fish products during storage at refrigeration temperatures.

1. Introduction

Fresh fish is consumed all over the world as one of the most nutritive food products. However, fish is highly perishable, even more than red meat or chicken, due to its large content of free aminoacids and volatile nitrogen bases compared with other meats [1]. The spoilage process of fish usually starts with the production of off-odors and flavours, generated basically by the growth of typical spoilage microorganisms. Besides, loss of texture and presence of slime could be detected. As a result of all these changes, the shelf-life of unpacked and packed fresh fish is very limited [1, 2]. Bacteria like *Shewanella* spp., *Pseudomonas* spp., *Aeromonas* spp., *Vibrio* spp., *Enterobacteriaceae*, and Lactic acid bacteria are common spoilers of fresh and chilled fish stored either under air or modified atmosphere conditions [3].

Hake *(Merluccius merluccius)* is one of the most consumed fish species in Spain, either fresh or frozen. In 2011, the 26% of consumed fresh fish in the Spanish market was hake. According to the Spanish Agricultural Ministry during 2013, the consumption per capita of fresh hake was 2.63 kg [4]. Therefore, any new strategy developed to preserve or extend the shelf-life of this fish species is considered important for this economic area.

Modified atmosphere packaging (MAP) has been extensively applied by the food industry as one of the most effective preservation techniques. MAP of fresh produce relies on modification of the gas composition inside the package, based on the interactions between two processes, the respiration of product, and the transfer of gases through the packaging [5]. According to results of previous works, modified atmosphere

in combination with refrigeration seems to be a suitable technology to extend the shelf-life of fresh fish and seafood [6, 7]. In a previous study, swordfish fillets were packaged under different atmospheres and stored under refrigeration. Results showed that MAP and vacuum packaging inhibited the growth of bacteria until day 9 of storage and the shelf-life was extended to 11-12 days [6]. In addition, Parlapani et al. [7] studied the microbiological changes of sea bass fillets stored under refrigeration and packed under air and commercial MAP. They found that fish samples stored under MAP had a shelf-life extension 4 days longer than samples stored under air. These two technologies retard the microbial activity but they do not fully control the deterioration of fish.

In addition to traditional methods used to extend the shelf-life of fish products, like MAP or chilling storage, there is an increasing interest in the use of edible films and coatings with antimicrobial properties in order to reduce, inhibit, or delay the growth of microorganism on the surface of foods. Edible films and coatings can be made of polysaccharides, proteins, and lipids and can act as carriers of different compounds like antioxidant, antimicrobials, and other preservatives in order to improve food quality and safety. Whey protein isolate (WPI) has been used as carrier of different natural preservatives (like essential oils) or synthetic one due to its ability to form flexible, odorless, and tasteless films [8]. Its effectiveness, when combined with essential oils (EOs), in reducing or inhibiting the growth of microorganisms in food products other than fish, has been demonstrated and it is well documented [9]. Fernández-Pan et al. [10] treated chicken breast fillets with WPI coatings incorporated with oregano and clove EOs and evaluated their effect against the microbiota developed during storage at refrigeration temperatures. They concluded that WPI-oregano coating duplicated the shelf-life of chicken samples. Zinoviadou et al. [11] wrapped beef cuts with WPI-oregano and thyme films, resulting in a significant reduction of total viable counts and a complete inhibition of lactic acid bacteria.

Oregano and thyme EOs have been widely tested *in vitro* as antimicrobial substances and effectively inhibited the growth of an extensive range of bacteria [12–14]. Phenolic compounds are the main active principles of EOs and the responsible for their antibacterial activity. The mode of action of phenolic compounds and in consequence of essential oils is associated with the ability of these compounds to harm and disintegrate the outer membrane of cells [15]. Thus, the use of EOs could be considered as part of a hurdle approach in combination with other preservation technologies such as MAP or edible coatings.

Multiple barrier technology, also known as hurdle technology, is a promising preservation strategy to give consumers safe products while keeping quality, freshness, and organoleptic aspects and extending their shelf-life. Hurdle technology makes use of existing or new different preservation techniques (also known as hurdles) effectively combined in order to achieve a multitarget and reliable microbial spoilage control [16]. Bacteria are stressed by the combination of hurdles, and synergistic or additive effects will be expected [17]. Therefore, the combined use of whey protein coatings, essential oils, refrigeration, and MAP, as different preservation technologies, could be an option for improving the microbiological quality of hake fillets due to the potential synergistic effect.

Then, the objectives of this work were (1) to evaluate the effectiveness of whey protein isolate edible coatings enriched with essential oils on the microbiological quality of hake fillets and (2) to assess the combined effects of edible coatings and MAP on the microbiological quality of hake fillets stored under refrigeration conditions. Besides, the influence of initial fish microbial loads in the effectiveness of edible coatings and MAP over the quality of hake fillets was analyzed.

2. Materials and Methods

2.1. Film Forming Solution Preparation. WPI (10% w/w) (Davisco Food International, USA) was dissolved in distilled water and 5% (w/w) of glycerol (Panreac Química, Spain) was added as plasticizer. Then, film forming solutions (FFS) were heated using a thermostatic bath at 90°C during 30 minutes under constant agitation. Food grade oregano (OR; 71,9% carvacrol, 4,28% thymol) and thyme (THY; 49,25% thymol, 18,99% p-cymene) EOs (Esencias Martínez Lozano, Spain) were added at 1 and 3% (w/w) once FFS were cooled at room temperature. The FFS were homogenized by ultrasonication (UP 400S Hielscher Ultrasound Technology, Germany) using a 7 mm diameter tip for 5 minutes at 100% of amplitude. During sonication FFS were maintained in an ice-water bath in order to avoid temperature raises over 40°C.

2.2. Fish Samples Preparation. Fresh boneless and skin-off fish fillets were obtained from a local store and transported to the laboratory in a cooler filled with ice. Under sterile conditions, fillets were cut into pieces of 5 × 3 × 2 cm (length × width × height) and 30 g weight approximately. Cut fillets were randomly separated into 6 groups: the first one without any treatment was used as control (CONTROL: C) and the other 5 groups were coated with the corresponding FFS. For coating, every piece was dipped in 150 ml of FFS for 1 minute; then the FFS excess was allowed to drip off for 45 seconds and finally pieces were dried for 5 minutes under air stream. After all this process, the pieces were dipped for a second time for 1 minute, drained for 45 seconds, and dried for 30 minutes under air stream. This two cycle coating process guaranteed the formation of a homogenous, uniform, and continuous coating in every fillet. Samples were stored at two packaging conditions: air packaging (Experiment 1) and modified atmosphere (50% CO_2/45% N_2/5% O_2) (Experiment 2) at 4°C during 8 days in the case of air packaging and 16 days in the case of modified atmosphere packaging. Both, control and coated samples were packaged in polypropylene trays, sealed with PE/PP/EVOH/PP film. The specific gas mixture was chosen based on previous studies found in literature [18, 19].

So, the samples prepared for Experiment 1 were as follows: CONTROL (C), Control-WPI (C-WPI), WPI-OR 1% (WPI-OR-1), WPI-OR 3% (WPI-OR-3), WPI-THY 1% (WPI-THY-1), and WPI-THY 3% (WPI-THY-3).

In the case of Experiment 2 the samples were as follows: CONTROL (C), Control-WPI + MAP (C-WPI-MAP), WPI-OR 1% + MAP (WPI-OR-1-MAP), WPI-OR 3% + MAP (WPI-OR-3-MAP), WPI-THY 1% + MAP (WPI-THY-1-MAP), and WPI-THY 3% + MAP (WPI-THY-3-MAP).

In order to evaluate if the initial microbial population of hake fillets affected the effectiveness of the treatments, a third experiment was prepared. Hake fillets were obtained from a local store which has daily provision of fresh fish direct from the main supplier. A special care with the hygiene of the fillets during cleaning and deboning processes was demanded from the new retail supplier. Samples were coated with WPI coatings containing 3% of oregano EO, packed under MAP and stored at 4°C during 12 days. The coating procedure was the same as that used for Experiments 1 and 2.

2.3. Microbiological Analysis. 25 g of sample was aseptically weighed, placed in a sterile plastic bag (Seward, UK), and homogenized with 225 ml of buffered peptone water (Cultimed, Spain) using a stomacher (Stomacher 400, England) for 2 minutes. Decimal dilutions were prepared as needed and seeded in the correspondent media in order to perform the following determinations: (a) total viable counts (TVC) on pour plates of Plate Count Agar (PCA, Merck-Germany) incubated for 48 hours at 30°C; (b) H_2S-producing bacteria (black colonies) on pour plates of Iron Agar (CONDA-Spain) incubated for 48 hours at 30°C; (c) total psychrotrophic bacteria on pour plates of Plate Count Agar (PCA, Merck-Germany) incubated for 7–10 days at 4°C; (d) *Enterobacteriaceae* on double-layered pour plates of Violet Red Bile Glucose (VRBG, Cultimed-Spain) incubated for 24 hours at 37°C; (e) *Pseudomonas* spp. on spread plates of Pseudomonas Agar Base (Oxoid-Spain) supplemented with Cetrimide-Fucidin-Cephalosporin (CFC, Oxoid-Spain) incubated for 48 hour at 30°C; and (f) lactic acid bacteria (LAB) on spread plates of de Man, Rogosa, and Sharpe Agar (MRS, CONDA-Spain) incubated for 5 days at 30°C. All plates were examined visually for typical colonies associated with each medium. Microbiological analyses were done at days 0, 4, 8, 12, and 16 of the storage period. All microbiological results are expressed as the log of the colony forming units (cfu) per gram of sample. All analyses were done in triplicate.

2.4. Statistical Analysis. All tests were performed in triplicate. Statistical analyses were conducted using SPSS 21.0 (IBM, USA) software. Significant differences among treatments were determined using ANOVA and Duncan's multiple range post hoc test (confidence level of 95%).

3. Results and Discussion

3.1. Microbiological Changes in Samples Packed under Air Conditions (Experiment 1). The microbial counts of samples packed under air conditions are shown in Figures 1 and 2. The initial value of TVC of hake fillets was higher (around 5 log cfu/g) compared with previous experiments performed with other fish species [6, 20]. This high load of bacteria could be related to poor handling practices during processing of fish fillets. On day 4 of analysis most of the samples reached 7 log cfu/g which is the threshold value defined by Foods [21] for the commercialization of fresh water and marine species.

Result showed that WPI-OR or WPI-THY samples with both concentrations of EOs did not show significant differences ($p < 0.05$) in TVC compared with the control samples at the end of the storage period (Figures 1(a) and 2(a)). Just a slight reduction of less than 1 log cfu/g was observed in WPI-THY-1 and WPI-THY-3 samples on day 4 of storage. No significant differences were also observed between C and C-WPI samples, indicating that WPI coatings did not have any effect (neither positive nor negative) on the microbiota of hake fillets.

LAB counts are shown in Figures 1(b) and 2(b). Comparing the final counts, no differences were observed for either WPI-THY-1 or WPI-THY-3 samples but a reduction of 1.5 log cfu/g was observed for WPI-OR-3 samples. Besides, significant reductions ($p < 0.05$) were observed in day 4 of treatment (2 and 1.5 log cfu/g, respectively, for WPI-OR-3 and WPI-THY-1 and WPI-THY-3).

Results of the evolution of *Enterobacteriaceae* are shown in Figures 2(c) and 3(c). Significant differences were observed among the 4 treatments (WPI + EOs at 2 concentrations) during the whole storage period. WPI-OR-3 and WPI-THY-3 reduced the bacteria load in 2 and 1 log cfu/g, respectively, at the end of the storage period (day 8). Similar reductions were observed also in day 4 of storage. Smaller but still significant differences were found in WPI-OR-1 samples.

The evolution of psychrotrophic bacteria during storage is shown in Figures 1(d) and 2(d). Similar to the case of TVC, none of the treatments significantly affected ($p < 0.05$) the growth of psychrotrophic bacteria. After day 4 of analysis, all counts reached 7 log cfu/g, the limit for microbiological quality of fish and seafood products advised by Foods [21].

H_2S producing bacteria (including *S. putrefaciens*) were significantly reduced for the treatments (Figures 1(e) and 2(e)). Reductions of about 2 log cfu/g were found in WPI-OR-3 samples. Reductions of 2 and 2.5 log cfu/g showed the WPI-THY-1 and WPI-THY-3 samples at the end of the storage period. WPI-OR-1 was not effective in reducing this bacteria.

Figures 1(f) and 2(f) showed the counts of *Pseudomonas* spp. This bacteria was not inhibited by WPI + OR treatment. On the other hand, a slight reduction in counts (0.5 log cfu/g) was observed in WPI-THY-3 samples at day 8 of storage. At the end of the storage period, final counts were >7.5 log cfu/g, showing that *Pseudomonas* spp. was the dominant spoiler of hake fillets, with counts almost similar to the TVC or psychrotrophic bacteria. In previous experiments done in our laboratory testing *in vitro* the antimicrobial activity of WPI films enriched with oregano and thyme EOs (results not shown) the high resistance of *Pseudomonas* spp. had been observed. It could be attributed to the high tolerance of this Gram-negative bacteria to the action of EOs [22, 23]. Mastromatteo et al. [24] tested sodium alginate films incorporated with thymol (one of the main constituents of oregano and thyme EO) on peeled shrimps and found, similar to our results, slight but significant reductions in counts of TV and psychrotrophic bacteria when using 1000 ppm of the compound. No reductions were found with coatings

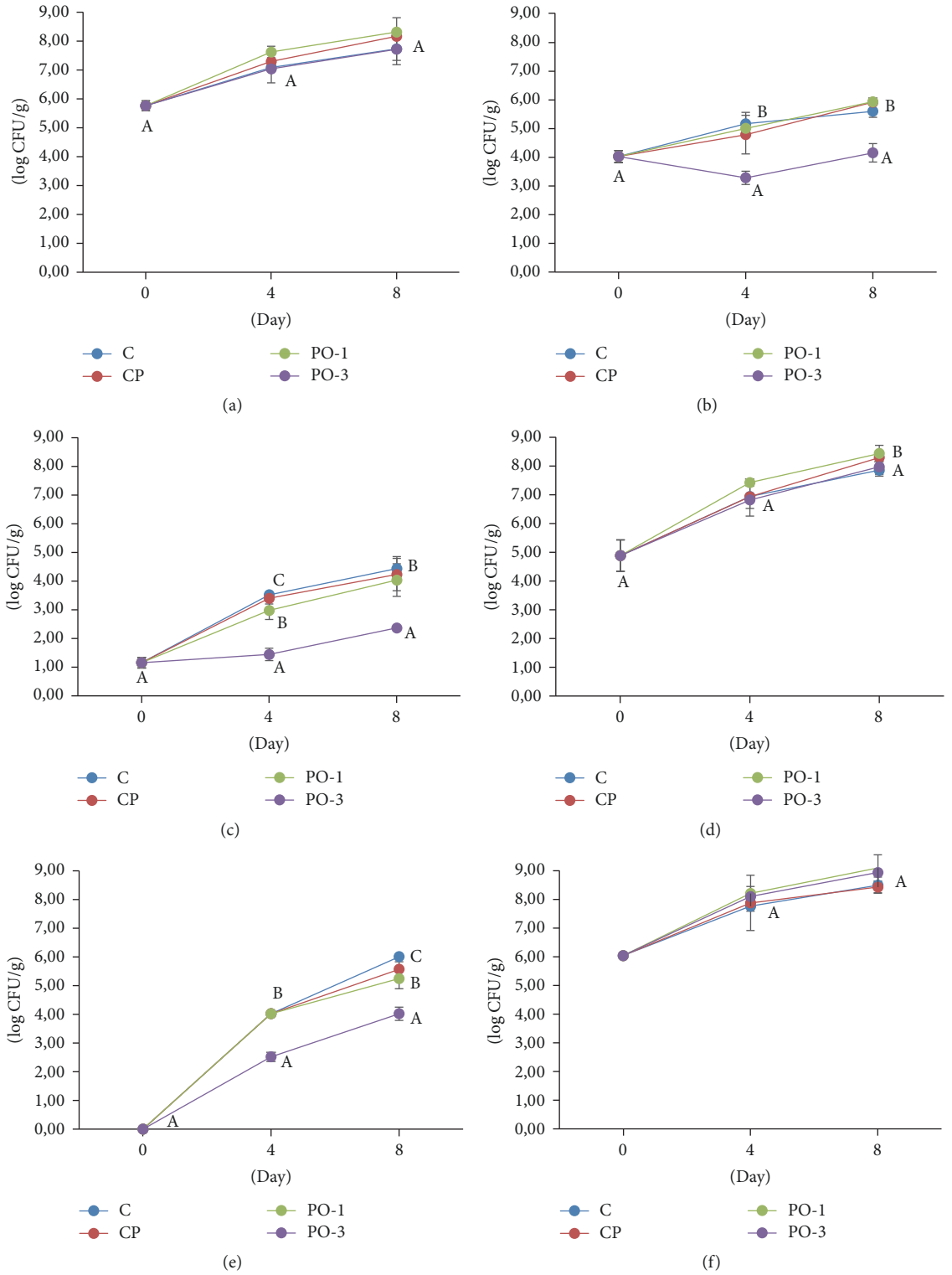

Figure 1: Evolution of the natural microbiota of WPI-Or coated hake fillet stored under air conditions. C: control, CP: control-WPI, PO-1: WPI + Or 1%, and PO-3: WPI-Or 3%. (a) Total viable counts; (b) lactic acid bacteria; (c) *Enterobacteriaceae*; (d) psychrotrophic bacteria; (e) H_2S- producing bacteria; (f) pseudomonas.

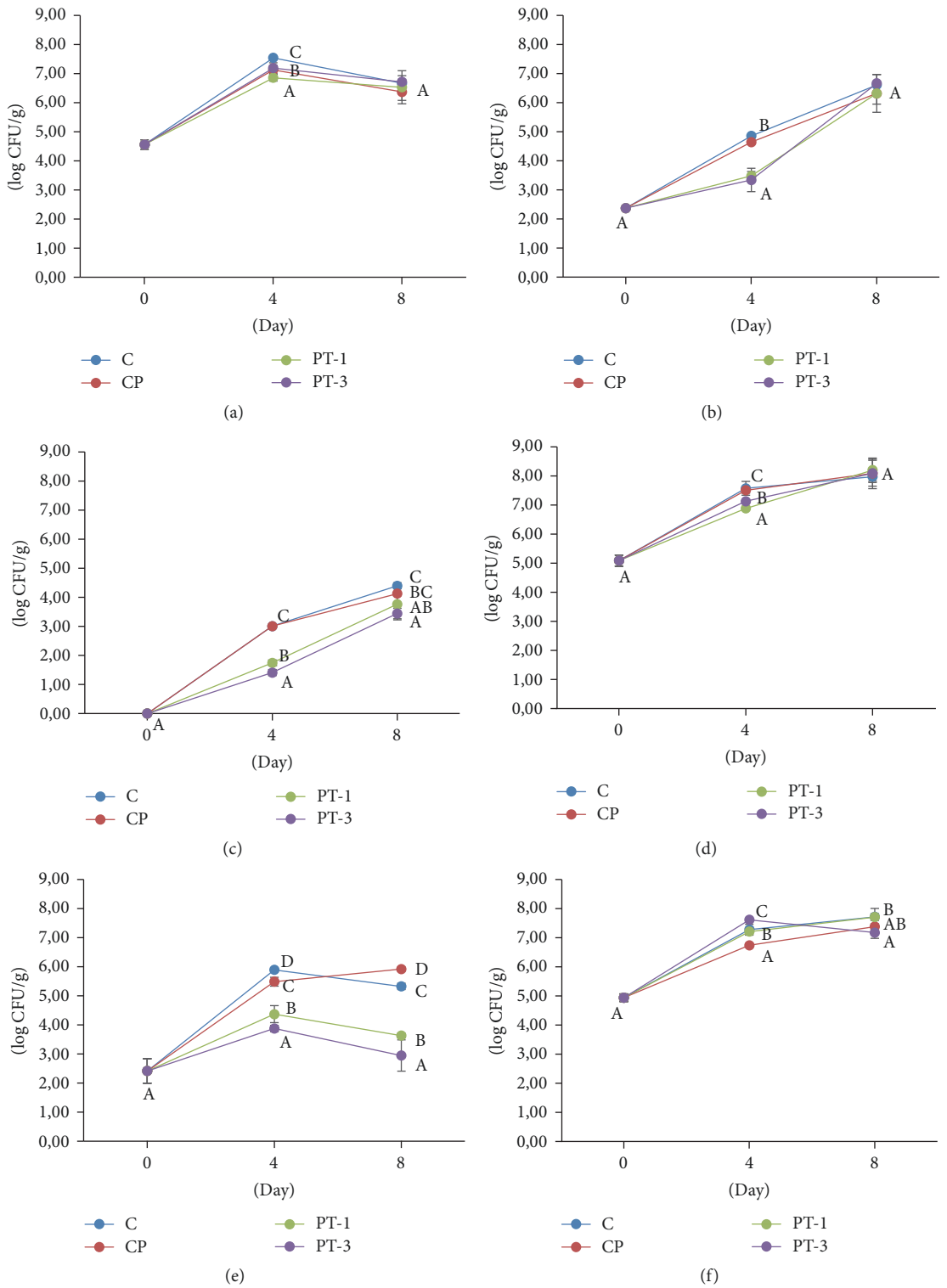

Figure 2: Evolution of the natural microbiota of WPI-Thy coated hake fillets stored under air conditions. C: control; CP: control-WPI; PT-1: WPI + Thy 1%; PT-3: WPI-Thy 3%. (a) Total viable counts; (b) lactic acid bacteria; (c) *Enterobacteriaceae*; (d) psychrotrophic bacteria; (e) H_2S- producing bacteria; (f) pseudomonas.

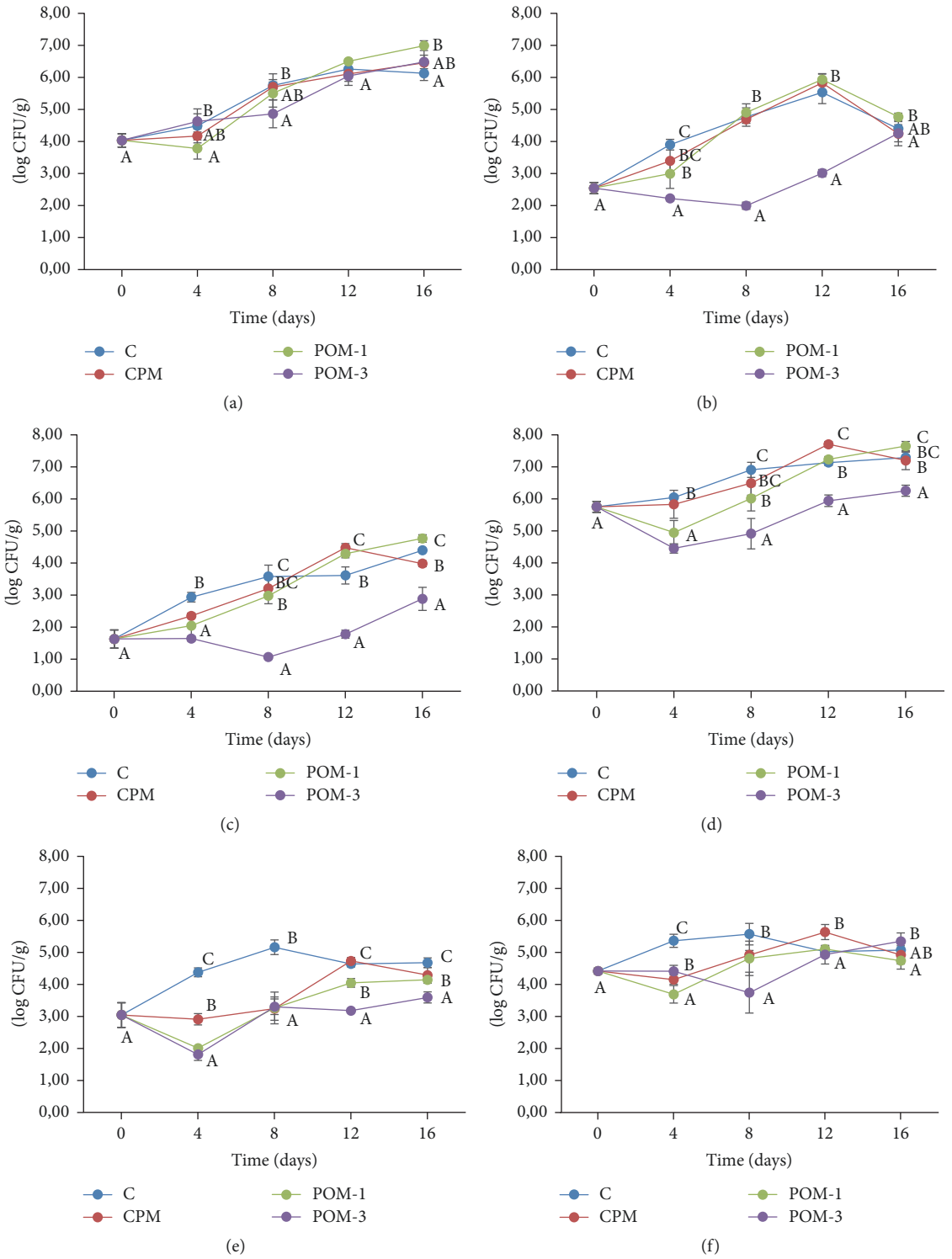

Figure 3: Evolution of the natural microbiota of WPI-Or coated hake fillets stored under MAP conditions. C: control; CPM: control-WPI; POM-1: WPI + Or 1%; POM-3: WPI-Or 3%. (a) Total viable counts; (b) lactic acid bacteria; (c) *Enterobacteriaceae*; (d) psychrotrophic bacteria; (e) H_2S- producing bacteria; (f) pseudomonas.

incorporated 500 ppm of thymol. Similar results have also been reported by Giatrakou et al. [25] with slight reductions in counts of TVC, *Pseudomonas* spp., H_2S-producing bacteria, and LAB and high reductions in *Enterobacteriaceae* when treating fresh swordfish with oregano EO. In a similar experiment, Zinoviadou et al. [11] treated fresh beef with WPI coatings incorporated with oregano EO and also found significant reductions in counts of TV and LAB during refrigerated storage.

3.2. Microbiological Changes in Samples Packed under MAP Conditions (Experiment 2). The microbial counts of samples packed under MAP conditions are shown in Figures 3 and 4. The evolution of TVC during the storage period for all treated samples stored under MAP conditions is shown in Figures 3(a) and 4(a). Initial counts were lower than the previous experiment (around 4 log cfu/g) which demonstrated a better quality of raw material [20, 26]. None of the samples examined reached the recommended value for TVC of 7 log cfu/g. No significant differences were observed between any of the investigated coatings ($p < 0.05$) with respect to control samples at the end of storage period, even though significant differences were observed at days 4 and 8 of storage, when reductions between 0.8 and 2 log cfu/g were found for WPI-OR-3-MAP and WPI-THY-3-MAP, respectively. Under this packaging condition, lower counts were observed compared to the values obtained for air packaged samples. This could be related either to the action of CO_2 as bacteriostatic agent or to lower initial counts in hake samples.

Counts of LAB are shown in Figures 3(b) and 4(b). No reductions were found at day 16 of storage period, but significant reductions were observed during the 12 first days of storage. For example, >2.5 log cfu/g reductions were observed in WPI-OR-3-MAP samples at day 8 of storage. Higher reductions were observed in WPI-OR-MAP samples when compared with WPI-THY-MAP samples in which in some cases (days 12 and 16 of storage) even no reductions were observed.

Enterobacteriaceae were successfully inhibited by all treatments (except WPI-OR-1-MAP) with significant reductions of 2 and 1 log cfu/g for WPI-OR-3-MAP and WPI-THY-3-MAP, respectively, at the end of the storage period. Slower growth of population of these microorganisms was observed during the 16 days of storage (Figures 3(c) and 4(c)). Even a decrease in counts in WPI-THY-1-MAP and WPI-THY-3-MAP samples was observed, indicating a clear inhibition of this bacterial group.

The same behavior as previous microorganisms was observed for psychrotrophic bacteria. No reductions in counts at day 16 were found for all treatments except for WPI-OR-3-MAP in which 1 log cfu/g reductions were observed. Significant differences in counts ($p < 0.05$) were observed during the storage period when comparing with the control samples, with reductions of 1.5 to 2.5 log cfu/g for both EOs (Figures 3(d) and 4(d)).

H_2S producing bacteria were also affected by the coating treatments. In this case, reductions of 1 and 2 log cfu/g were found at day 16 (WPI-OR-3-MAP and WPI-THY-3-MAP, respectively). Also during the whole storage period, significant and similar reductions were observed for the aforementioned treatments (Figures 3(e) and 4(e)).

In the case of *Pseudomonas* spp. the final population was slightly reduced for WPI-OR-1-MAP and WPI-THY-1-MAP, while the treatments effectively decreased the counts at days 4, 8, and 12 of storage, with reductions ranging from 1 to 2 log cfu/g. Final counts of *Pseudomonas* spp. were <5.5 log cfu/g, which are very low values compared with previous studies (and also with Exp. 1), in which counts used reached values higher than 7 or 8 log cfu/g [6, 27].

Similar to our results, reductions in TVC, H_2S producing bacteria, and *Pseudomonas* spp. have been reported by Kykkidou et al. [19] who treated swordfish fillets with 0.1% thyme EO and stored the samples under MAP conditions. A combination of oregano EO and MAP was also tested by Pyrgotou et al. [26] on the shelf-life of rainbow trout fillets for a period of 21 days. Significant reductions were found in TVC, *Pseudomonas* spp., H_2S producing bacteria, LAB, and *Enterobacteriaceae* resulting in a shelf-life extension of 8 days.

For all the microorganisms tested a synergistic effect could be observed until day 12 of storage. Samples treated with WPI + EOs + MAP were significantly different when compared with control samples (just MAP packed) and sample treated just with WPI coating without the presence of EOs + MAP. In all the cases higher reductions were observed in samples packaged under MAP conditions and coated. Furthermore, the effectiveness of the treatments is clearly dependent on the microbial group analyzed. Although in some cases the counts for all the treatments were similar after 16 days of storage and no differences were detected, the microorganisms were effectively inhibited within the first 8 or 12 days of storage. This could be because (a) the EOs included in the coatings lost activity during the storage period due to evaporation from the coating; (b) the microorganisms were higher in number and the EOs were not able to exert their mode of action; or (c) the amount of CO_2 remained in the package could be lower than the initial value; then it was not enough to act as a preservative.

3.3. Microbiological Changes in Samples with Low Initial Microbial Load (Experiment 3). Comparing the initial counts of fresh hake fillets for Exp. 1 and Exp. 2, differences can be observed. TV and psychrotrophic counts in Exp. 1 are, respectively, 1.5 and 1 log cfu/g higher than in Exp. 2. Besides, when comparing final counts (in samples stored under air) for the same bacterial groups at day 8 of storage, differences in counts remained as high as the initial one. Then, the poor effectiveness of WPI coating treatments found in Exp. 1 could be attributed to the high initial microbial population. Therefore, in order to verify if the initial bacterial counts of samples could affect the effectiveness of WPI coatings, a new experiment was performed. Four lots of new samples obtained from a new supplier (with low initial counts) were coated as before, having the following treatments: CONTROL (C), Control-MAP (C-MAP), WPI-OR 3% (WPI-OR-3), and WPI-OR 3% + MAP (WPI-OR-3-MAP).

Microbiological analyses were done at days 0, 4, 8, and 12 of the storage period. Besides, the possible, but still not clear,

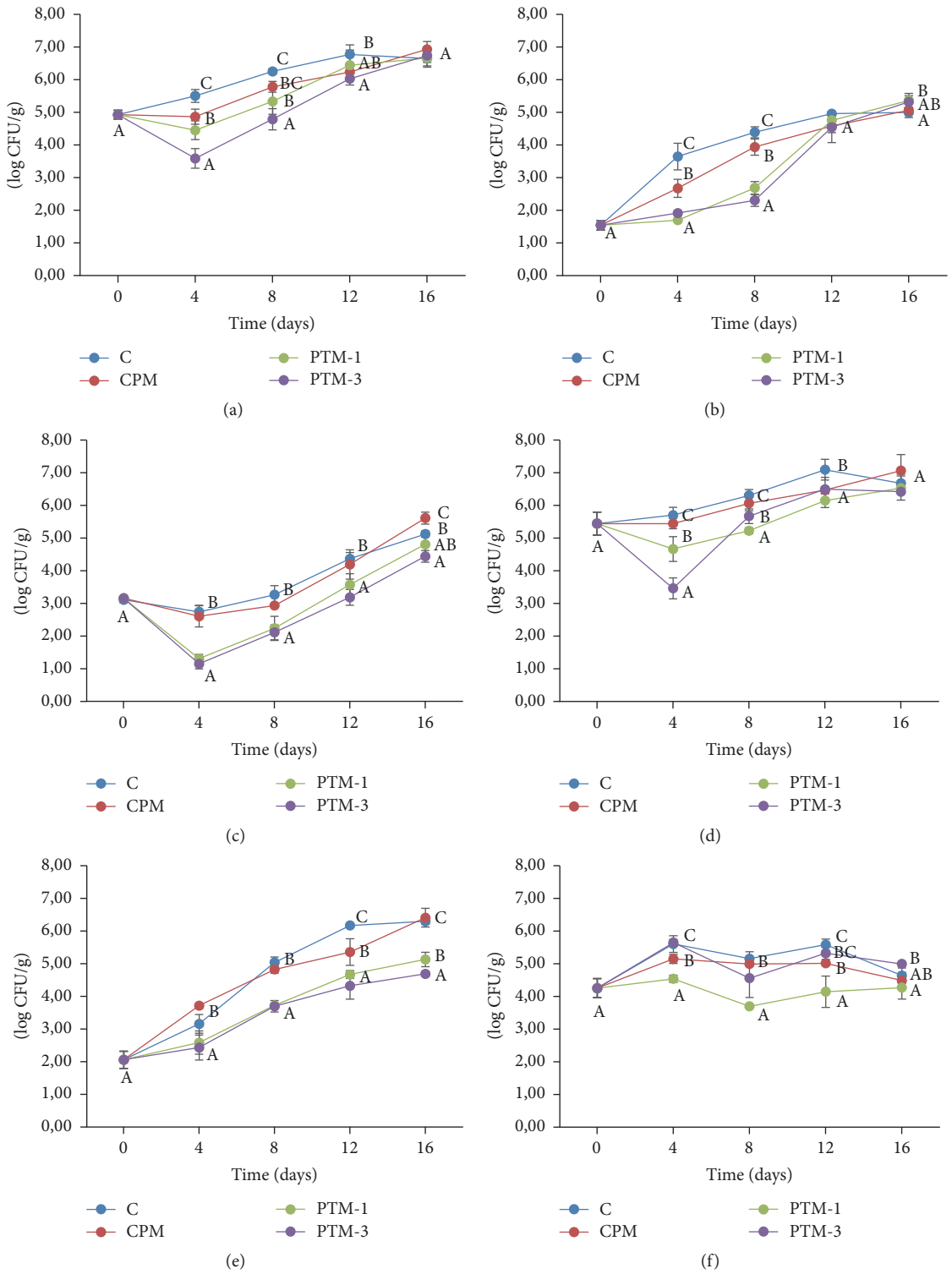

Figure 4: Evolution of the natural microbiota of WPI-Thy coated hake fillets stored under MAP conditions. C: control; CPM: control-WPI; PTM-1: WPI + Thy 1%; PTM-3: WPI-Thy 3%. (a) Total viable counts; (b) lactic acid bacteria; (c) *Enterobacteriaceae*; (d) psychrotrophic bacteria; (e) H_2S- producing bacteria; (f) pseudomonas.

synergistic effect between WPI coatings and MAP, observed in Exp. 2, was also verified.

Low microbial counts were found in day 0 of analysis (Figure 5). Around 4 log cfu/g were registered for TVC and psychrotrophic bacteria. *Pseudomonas* spp. were around 3 log cfu/g. *Enterobacteriaceae*, LAB, and H_2S-producing bacteria were undetectable (<1 log cfu/g). Other authors have reported also low counts at the beginning of the experiment, showing better and acceptable microbiological quality [27, 28].

Changes in TVC of treated hake fillet samples during storage are shown in Figure 5(a). Significant differences ($p < 0.05$) were found in the counts of all treated samples. C, C-MAP, and WPI-OR-3 samples exceeded the threshold recommended for commercialization of fresh marine species [21] at days 8 and 12 of storage, respectively. On the other hand, WPI-OR-3-MAP samples did not reach the recommended value at any point of the 12 days of storage (final counts of 5.64 log cfu/g). This result indicated that the combination of coating and MAP technology was much more effective together. When WPI + OR coating was used in combination with MAP, a significant inhibition of TV counts was observed, with >3 log cfu/g reduction at day 12 of storage. Similar results have been reported in previous works [25, 29, 30]. Despite the final values of C-MAP and WPI-OR-3 samples exceeding the threshold, significant reductions were found during the whole storage period, having higher reductions in C-MAP samples compared with WPI-OR-3 samples. From Figure 5(a), an extension of the lag phase could be observed in all samples except the control. Counts of C samples increased steadily from day 4 while the TVC growth rate was lower in the other cases.

LAB are part of natural microbiota of hake fillets and as facultative anaerobic bacteria can grow under high concentration of CO_2, thus constituting an important part of the microbiota of fresh fish packed under MAP conditions. In the current study the initial LAB counts were not detectable (Figure 5(b)) and at the end of the storage period counts reached 6 log cfu/g for C samples. Significant differences were observed when comparing treatments ($p < 0.05$). At day 12, C-MAP treatment was unable to inhibit the growth of LAB while WPI-OR-3 decreased the counts in 1 log cfu/g compared with C. The highest inhibition was observed in WPI-OR-3-MAP samples with 2.5 log cfu/g counts lower than the control samples. An extension of the lag phase it is also observed in Figure 5(b). The same Figure also showed that LAB growth rate in WPI-OR-3-MAP samples during the storage was lower than the other samples. Gram-positive microorganisms like LAB are generally more resistant to frozen storage, salt, low water activity, and high carbon dioxide levels than Gram-negative microorganisms [31]. On the other hand, it is well known that these groups of microorganisms are sensitive to the action of EOs [23, 32]. For these reasons, a higher and a synergistic effect of the combined use of EO incorporated in edible films and MAP conditions are expected.

Enterobacteriaceae were undetectable at days 0 and 4 of analysis (Figure 5(c)) indicating a good hygiene and acceptable handling practices of the hake fillets. WPI-OR-3 and WPI-OR-3-MAP treatments had a significant effect on the samples since complete inhibition of this bacteria was observed through the experiment. A reduction of around 1 log cfu/g was observed in the case of C-MAP samples when compared with the final counts of C samples. The population of *Enterobacteriaceae* was lower compared with other microorganisms' groups analyzed in this study; however, the contribution of this bacteria to the final microbiological quality has to be taken into account.

There is a clear and significant effect of WPI-OR coatings alone or in combination with MAP on the growth of LAB. Similar results were found by Gómez-Estaca et al. [30] who treated cod fillets with chitosan-clove films and found total inhibition of this bacteria at the end of the storage period. Pantazi et al. [6] also found reductions in *Enterobacteriaceae* counts when analyzing fresh swordfish packed under different packaging conditions. MAP samples had lower counts compared with samples stored under air conditions.

With a similar behavior as TVC, significant differences ($p < 0.05$) among samples were shown for psychrotrophic bacteria counts. Reductions of 3, 1.5, and 0.5 log cfu/g were observed for WPI-OR-3-MAP, WPI-OR-3, and C-MAP samples, respectively (comparing with C), having the higher inhibitory effect, once again, in samples coated and packaged under MAP conditions (Figure 5(d)). Similar results were found by López de Lacey et al. [33] who showed that agar films containing green tea extract delayed the growth of psychrotrophic bacteria, including *Pseudomonas* spp. and H_2-S microorganisms. In their study Mastromatteo et al. [24] also found differences in psychrotrophic bacteria counts when comparing peeled shrimp treated with alginate coatings enriched with thymol and packed under MAP and stored under air conditions.

H_2S producing bacteria including *S. putrefaciens* like-bacteria are normally used as a good spoilage indicator of fish and seafood products [34]. At the beginning of the experiment H_2-S producing bacteria counts were not detected but later exponential growth was observed in all samples. Counts for WPI-OR-3-MAP, C-MAP, and WPI-OR-3 samples were significantly ($p < 0.05$) lower than for C samples during the whole storage period (Figure 5(e)). Differences in counts of 3, 2.5, and 2 log cfu/g, respectively, were detected at day 12, when compared with control samples. Similarly Boskou and Debevere [35] reported that *S. putrefaciens* was unable to develop when high concentration of CO_2 (>50%) was applied on cod fillets. Ravn Jørgensen et al. [36] concluded that the growth of *S. putrefaciens* is inversely related to the shelf-life of iced cod, meaning higher counts shorter shelf-life. In addition Gram et al. [37] reported that counts of *S. putrefaciens* higher than 6 log cfu/g start producing sulfur compounds and spoilage of fish takes place. In our case, lower values of H_2-S producing bacteria (4 log cfu/g) were observed for WPI-OR-3-MAP samples and are related to the lower values obtained for TVC (5.64 log cfu/g) and psychrotrophic bacteria (5.80 log cfu/g), confirming the shelf-life extension of hake fillets. Final counts of 7.25 log cfu/g for H_2-S producing bacteria in C samples were registered.

Pseudomonas spp. together with *Shewanella* spp. are common spoilers of chilled fish and seafood products packed

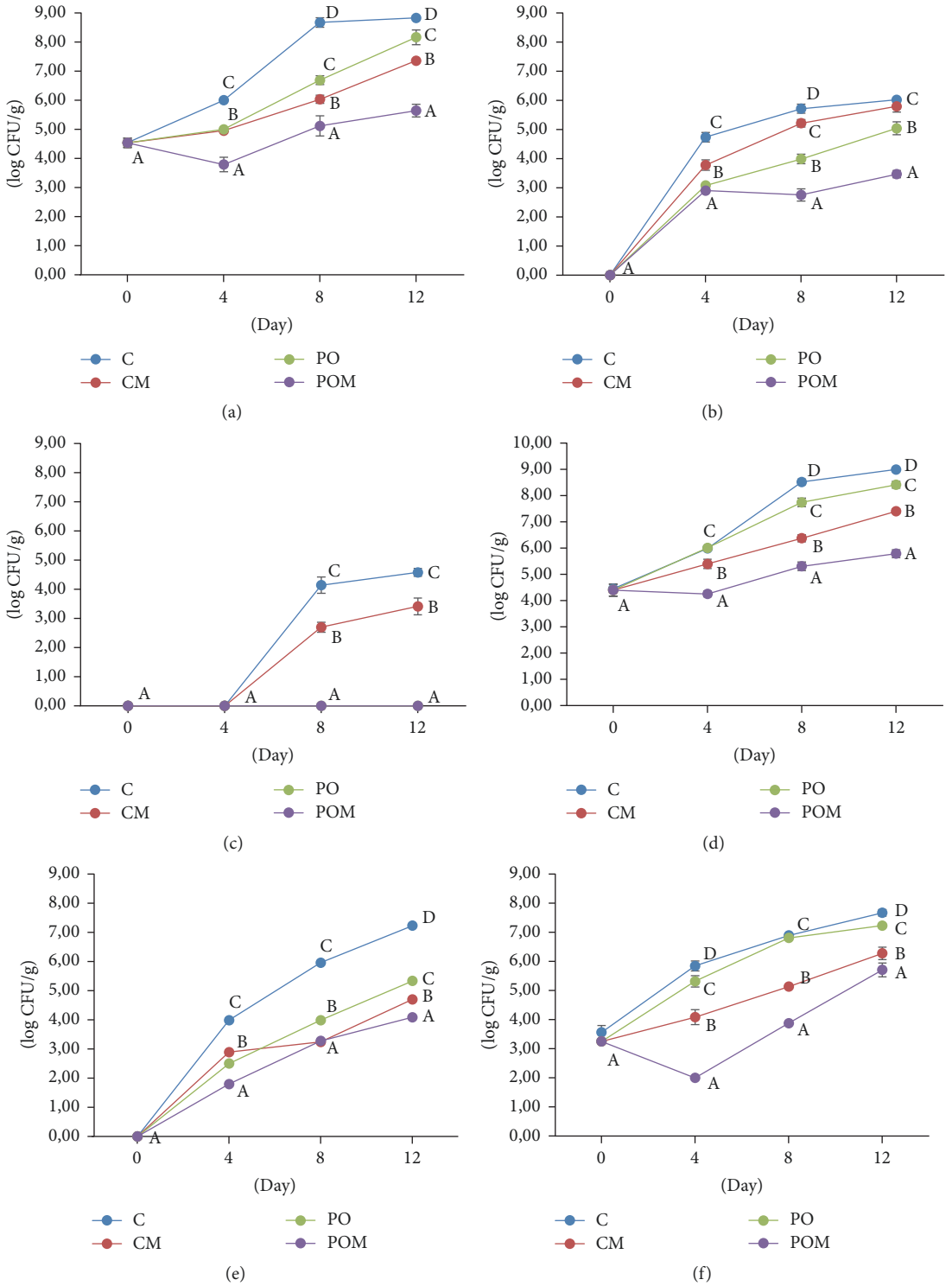

Figure 5: Evolution of the natural microbiota of WPI-Or coated hake fillet stored under air and MAP conditions. C: control, CP: control-WPI, PO-1: WPI + Or 1%, PO-3: WPI-Or 3% ($p < 0.05$). (a) Total viable counts; (b) lactic acid bacteria; (c) *Enterobacteriaceae*; (d) psychrotrophic bacteria; (e) H_2S- producing bacteria; (f) pseudomonas.

under aerobic conditions [31]. They have also been reported as specific spoilage microorganisms or fish from temperate water [34]. WPI-OR-3-MAP samples presented the lowest counts (5.71 log cfu/g) when compared with the other treatments and C samples. Figure 5(f) shows the evolution of *Pseudomonas* spp. during the storage period. At the end of the storage time the final counts reached 7.67 log cfu/g for C samples. *Pseudomonas* spp. presented at the end of the storage period higher counts for all samples than H_2-S producing bacteria or other bacteria group, therefore constituting the major flora of hake fillets. Similar results were found in previous experiments with different fish species like cod [30], swordfish [6], or sole [27] where *Pseudomonas* spp. were also the predominant bacteria.

According to Spanish law (RD 135/2010 B.O.E. 25/02/2010) the limit for commercialization of fresh fish is 7 log cfu/g for TVC and 4 log cfu/g for *Enterobacteriaceae*. Comparing these two data for control and treated samples, a shelf-life extension of 4 days for C-MAP samples and >4 days for WPI-OR-3-MAP hake samples was achieved, attributed to the combination of the antimicrobial effect exerted by the WPI-oregano EO coating and the MAP conditions. Besides, the effect of low initial counts was also observed, being a key parameter for the potential application of edible coatings.

4. Conclusions

In Exp. 1, when samples with high initial bacteria load were used, WPI coatings incorporated with oregano and thyme EOs at different concentrations did not affect the microbial growth of most of the microorganisms developed in fresh hake fillets packed under air and stored for 8 days at refrigeration conditions. In Exp. 2 when active coatings were combined with MAP conditions (but still high initial counts in samples), same bacterial group as before were inhibited but higher antimicrobial effect was observed during the storage period.

Results of Exp. 3 showed that the combination of EO-enriched coatings with MAP and low initial microbial counts was effective in improving the microbiological quality of fresh hake fillets. While in Exp. 1 coatings did not improve the quality of the samples, in Experiment 3 (working with lower initial microbial population) the shelf-life of hake fillets treated with WPI + OR 3% + MAP was doubled (from the microbiological point of view). The growth of all microorganisms analyzed was delayed, especially H_2S-producing bacteria. The antimicrobial effectiveness was clearly dependent on the initial microbial population, the concentration of EO, the presence of MAP, and the bacteria genera.

The synergistic effect of this combined strategy was observed. Applying both EO, incorporated into WPI coatings, and high concentration of CO_2 was more effective in maintaining the quality of hake fillets than applying only WPI-EO or MAP. Such effect was characterized by lower microbial counts and extension of the lag phase.

Summarizing, the application of WPI-enriched films combined with MAP conditions delayed the growth of microorganism in hake fillets. The low initial microbial load of samples seems a key parameter to take into consideration. The combination of WPI edible coatings with oregano and thyme EO and a packaging atmosphere rich in CO_2 could be a valid alternative to preserve fish and fish products.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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